

Mechanistic assessment of peptide reactivity assay to predict skin allergens with Kathon[®] CG isothiazolinones

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ABSTRACT

Assessment of skin sensitization hazard of chemicals currently depends on in vivo methods. Considering the forthcoming European Union ban on in vivo testing of cosmetic/toiletry ingredients, the search for alternative non-animal approaches is an urgent challenge for investigators today. For the skin sensitization end-point the concept of protein/peptide haptentation, that could reflect the chemical modification of skin proteins, crucial to form immunogenic structures, has been used to develop in vitro assays to predict the sensitization potential of new chemicals. Using glutathione and nucleophile-containing synthetic peptides we confirmed previously the possibility to screen for skin sensitization potential by measuring peptide depletion following incubation with a set of allergens and non-allergens. In this paper, additionally to our model development work, we performed mechanistic based studies to confirm the peptide reactivity concept under the specific conditions used for haptens in the screening assay as they were somewhat different from the ones expected to happen in vivo. Following the reactivity toward the peptides of ¹³C labelled MI and MCI, models of true haptens, we showed that the initial step leading to the biological end-point was similar regardless the conditions used even if final adducts could be different. This confirmed the validity of the peptide reactivity concept as well as the choice made to look at peptide depletion rather than at adduct formation.

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1. Introduction

Allergic contact dermatitis (ACD) is an immunologically based disease resulting from skin sensitization to a chemical. The sensitization process starts by the chemical modification of epidermal proteins by the allergen or hapten. Processing of the hapten–protein complex by immunocompetent skin antigen-presenting cells ensures the presentation of the altered peptides to naïve T-lymphocytes in the lymph nodes. This process results in the selection and activation of T-lymphocyte sub-populations with T-cell receptors specific for the chemical modification that will be activated following a second contact with the allergen. Individuals sensitized this way to the chemical are predisposed to develop ACD, characterized by the appearance of erythema and oedema, at the site of

subsequent skin exposure to the same or cross-reactive chemicals (Rustemeyer et al., 2006).

Exposure to allergens and the subsequent risk of contact sensitization has become an essential regulatory issue within industry in the last few years. At present, the only validated approaches to identify sensitization hazards are in vivo models such as the local lymph node assay (LLNA) (Kimber et al., 1994). As a consequence of legislative initiatives such as registration, evaluation, authorization and restriction of chemicals (REACH; European Commission, 2006), and the seventh amendment to the European cosmetics directive, which poses a ban on animal testing for cosmetic ingredients (European Commission, 2003), an urgent challenge for investigators today is to develop non-animal based methods for the evaluation of the skin sensitization potential of new chemicals.

A key step in the sensitization process is the formation of a covalent adduct between the sensitizer and skin proteins (Lepoittevin, 2006). Since this reactivity is a critical step in the induction of skin sensitization, it was hypothesised that an in vitro method to screen the sensitization potential of chemicals could be based on the measure of peptide reactivity. In the 30s it was established that a correlation exists between the skin sensitization potential of a chemical and its reactivity toward nucleophiles (Landsteiner and Jacobs, 1936). Indeed the majority of allergens

Abbreviations: ACD, allergic contact dermatitis; MCI, 5-chloro-N-methyl-isothiazolin-3-one; MI, N-methyl-isothiazolin-3-one; GSH, glutathione; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum correlation; LLNA, local lymph node assay; PBS, phosphate buffer solution; Pep-Cys, cysteine peptide; Pep-Lys, lysine peptide; REACH, registration, evaluation, authorization and restriction of chemicals.

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have electrophilic properties and are able to react with nucleophilic amino acids in proteins to form covalent bonds. To assess if this approach could be used as a skin sensitization screening tool, we developed a peptide based assay in which the reactivity of chemicals of different allergenic potencies (weak to extreme, along with non-sensitizers) toward glutathione (GSH) and synthetic peptides containing nucleophilic amino acids such as cysteine (Pep-Cys) or lysine (Pep-Lys) was measured (Gerberick et al., 2004). After 15 min reaction time for GSH at a 1:100 peptide to chemical ratio, and 24 h reaction time for the synthetic peptides at 1:10 and 1:50 peptide to chemical ratios, the samples were analyzed by HPLC using UV detection to monitor the peptides depletion following incubation with the chemicals. Initial results with 38 compounds indicated a significant correlation between allergen potency and the peptides depletions. Generally, moderate, strong and extreme sensitizers showed moderate to high reactivity, while weak and non-sensitizers showed minimal to low reactivity. The analysis was expanded by evaluating a total of 82 chemicals (Gerberick et al., 2007). The peptide reactivity data were compared with existing LLNA data using recursive partitioning methodology to build a classification tree that allowed a ranking of reactivity as minimal, low, moderate and high. A model based on Pep-Cys at 1:10 and Pep-Lys at 1:50 was proposed to rank reactivity using the approach of measuring peptide depletion at a fixed time and concentration. Classifying minimal reactivity as non-sensitizers and low, moderate and high reactivity as sensitizers, this model gave a prediction accuracy of 89%.

These studies confirmed that measurement of peptide reactivity is worth to be considered as a screening approach for skin sensitization testing. Still, the reaction conditions used for the peptide depletion test were quite different from the ones suspected to happen in vivo. In several reactivity studies reported in the literature, haptens were reacted with models of nucleophiles, amino acids, and peptides in excess (10–100-folds) as these are the conditions supposed to happen in vivo (Alvarez-Sánchez et al., 2004a; Eilstein et al., 2006). It was demonstrated on several examples that, under these conditions, adducts formed with proteins were identical to the ones formed with model nucleophiles, amino acids or short peptides, and it was postulated that the reactions taking place with proteins were a good model of the sensitization mechanism. On the contrary, in the peptide reactivity based screening assay an excess of hapten was used in order to promote maximum peptide depletion. Therefore, to support the developed screening test for skin sensitization, it was necessary to confirm the peptide reactivity concept under the specific conditions used for haptens. Moreover, the choice of measuring peptide depletion rather than the formation of covalent adducts needed to be evaluated.

In this work we compared mechanisms leading to the depletion of the test peptides and to the formation of adducts under the screening assay conditions (excess of hapten) and under the conditions supposed to happen in vivo, called from now classical reactivity conditions (excess of peptide). *N*-Methyl-isothiazolin-3-one (MI) and 5-chloro-*N*-methyl-isothiazolin-3-one (MCI), two widely used preservatives, were selected as models of true haptens, weak and strong respectively. Evidence of contact allergy to the MI/MCI mixture, active ingredient of the biocide Kathon® CG used in the cosmetics industry, has been deduced from experimental studies in animals and humans, from observations in industry and from accumulated case reports associated with the presence of these compounds in consumer products (Fewings and Menné, 1999). In addition, patch test studies carried out on patients sensitized to Kathon® CG using serial dilutions of pure MI and MCI showed that the chlorinated derivative MCI was the stronger sensitizer, while the non-chlorinated derivative MI was a much weaker sensitizer (Bruze et al., 1987a,b). These results were corroborated by the LLNA

data reported in the literature for isolated MI and MCI (Gerberick et al., 2005).

Reaction mechanisms and adducts formation toward GSH, Pep-Cys and Pep-Lys were investigated using ^{13}C labelled MI and MCI and mono- and two-dimensional NMR. Results indicated a similar initial chemistry step in all experimental conditions, but also the existence of multi-step reactions leading to the formation of many adducts with different structures. This confirmed the validity of the peptide reactivity concept but also that a quantification of adducts would be difficult and probably misleading while a measure of the depletion is a good approach to the assessment of reactivity of these molecules toward nucleophiles for the purpose of screening for skin sensitization.

2. Materials and methods

2.1. Chemicals and reagents

4- ^{13}C -MI and 4- ^{13}C -MCI (Fig. 1) were synthesised using a procedure we had reported previously (Alvarez-Sánchez et al., 2003). GSH was purchased from Sigma–Aldrich (Saint Quentin Fallavier, France) and used without further purification. Heptapeptides containing lysine (*N*-Ac-RFAAKAA-COOH; Pep-Lys) and cysteine (*N*-Ac-RFAACAA-COOH; Pep-Cys) were prepared by the SynPep Corporation (Dublin CA, USA) and by the Proteins Platform of the Institut Fédératif de Recherche en Neurosciences (Strasbourg, France). They were purified >90% by HPLC. Molecular weight confirmation was done by mass spectrometry with electrospray ionization in the positive mode. Deuterated acetonitrile and water were obtained from Euriso-Top (Saint Aubin, France). Spectrophotometric grade dimethylsulfoxide and acetonitrile, and sodium phosphate monobasic, sodium phosphate dibasic and ammonium acetate for the preparation of buffers were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France).

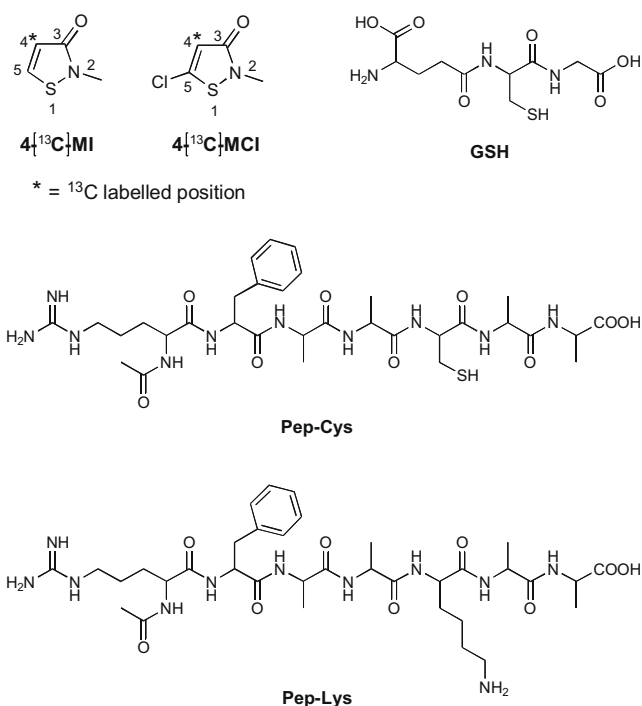


Fig. 1. Chemical structures of 4- ^{13}C -MI, 4- ^{13}C -MCI, GSH and the synthetic peptides containing cysteine or lysine as nucleophilic amino acids.

2.2. Reaction of 4-[¹³C]-MI and 4-[¹³C]-MCI toward an excess of peptides (classical reactivity conditions)

General procedure: to the 4-[¹³C]-N-methyl-isothiazolone, dissolved in CD₃CN (260 μL), was added the peptide in excess (GSH, 10 equiv.; Pep-Cys and Pep-Lys, 2 equiv.) dissolved in deaerated deionized water (390 μL). The solution was filtered into an NMR tube, and the reaction was followed by ¹³C NMR.

In particular, for each experiment: MI (0.5 mg, 4.3 μmol) and GSH (13.2 mg, 43.0 μmol, 10 equiv.); MI (0.5 mg, 4.3 μmol) and Pep-Cys (6.5 mg, 8.6 μmol, 2 equiv.); MI (0.5 mg, 4.3 μmol) and Pep-Lys (6.7 mg, 8.6 μmol, 2 equiv.); MCI (1 mg, 6.64 μmol) and GSH (20.4 mg, 66.4 μmol, 10 equiv.); MCI (0.5 mg, 3.32 μmol) and Pep-Cys (5 mg, 6.64 μmol, 2 equiv.); MCI (0.5 mg, 3.32 μmol) and Pep-Lys (5.2 mg, 6.64 μmol, 2 equiv.).

2.3. Reaction of an excess of 4-[¹³C]-MI and 4-[¹³C]-MCI toward the peptides (peptide depletion test conditions)

General procedure: to the 4-[¹³C]-N-methyl-isothiazolone, dissolved in the minimum amount of d₆-DMSO or CD₃CN (10 μL), was added the peptide dissolved in a buffer solution (477 μL). D₂O (163 μL) was then added to complete the final volume of the reaction solution (650 μL). This one was filtered into an NMR tube, and the reaction was followed by ¹³C NMR.

In particular, for each experiment: MI (1 mg, 8.61 μmol, 2 equiv.; 3 mg, 25.83 μmol, 10 equiv.), GSH (1.3 mg, 4.3 μmol for 2 equiv. MI; 0.8 mg, 2.58 μmol for 10 equiv. MI) dissolved in phosphate buffer (0.13 M, pH 7.8) and D₂O (163 μL; final buffer solution in the NMR tube: 0.1 M, pH 7.4); MI (1 mg, 8.61 μmol, 2 equiv.; 1 mg, 8.61 μmol, 10 equiv.), Pep-Cys (3.2 mg, 4.3 μmol for 2 equiv. MI; 0.7 mg, 0.86 μmol for 10 equiv. MI) dissolved in phosphate buffer (0.13 M, pH 7.9) and D₂O (163 μL; final buffer solution in the NMR tube: 0.1 M, pH 7.5); MI (1 mg, 8.61 μmol, 2 equiv.; 1 mg, 8.61 μmol, 10 equiv.), Pep-Lys (3.3 mg, 4.3 μmol for 2 equiv. MI; 0.7 mg, 0.86 μmol for 10 equiv. MI) dissolved in ammonium acetate buffer (0.1 M, pH 10.2) and D₂O (163 μL; final buffer solution in the NMR tube: 0.1 M, pH 10.2); MCI (1 mg, 6.64 μmol, 2 equiv.; 3 mg, 19.92 μmol, 10 equiv.), GSH (1 mg, 3.32 μmol for 2 equiv. MCI; 0.6 mg, 1.99 μmol for 10 equiv. MCI) dissolved in phosphate buffer (0.13 M, pH 7.8) and D₂O (163 μL; final buffer solution in the NMR tube: 0.1 M, pH 7.4); MCI (1 mg, 6.64 μmol, 2 equiv.; 1 mg, 6.64 μmol, 10 equiv.), Pep-Cys (2.5 mg, 3.32 μmol for 2 equiv. MCI; 0.5 mg, 0.66 μmol for 10 equiv. MCI) dissolved in phosphate buffer (0.13 M, pH 7.9) and D₂O (163 μL; final buffer solution in the NMR tube: 0.1 M, pH 7.5); MCI (1 mg, 6.64 μmol, 2 equiv.; 1 mg, 6.64 μmol, 10 equiv.), Pep-Lys (2.6 mg, 3.32 μmol for 2 equiv. MCI; 0.5 mg, 0.66 μmol for 10 equiv. MCI) dissolved in ammonium acetate buffer (0.1 M, pH 10.2) and D₂O (163 μL; final buffer solution in the NMR tube: 0.1 M, pH 10.2).

2.4. NMR experiments and structure assignment

The reactions were followed by mono-dimensional ¹³C NMR on a Bruker Avance 300 spectrometer at 75 MHz. The structure of the products formed in the reactions was elucidated by two-dimensional [¹H–¹³C]-NMR. Heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple-bond correlation (HMBC) experiments were recorded on Bruker Avance 400 (¹H, 400 MHz; ¹³C, 100 MHz) and Bruker Avance 500 (¹H, 500 MHz; ¹³C, 125 MHz) spectrometers. Chemical shifts (δ) are reported in ppm with respect to TMS, using acetonitrile (¹H, δ = 2.06; ¹³C, δ = 119.7) or dimethylsulfoxide (¹H, δ = 2.52; ¹³C, δ = 39.4) as internal standard. Structures of the different adducts were assigned using a combination of the HSQC and the HMBC data obtained. The measured chemical shifts were compared with those calcu-

lated using the additivity principle and NMR data derived from analogous compounds by using ACD/CNMR and ACD/HNMR Predictor software (version 5.12).

3. Results

MI and MCI ¹³C labelled at position C-4 were used in these studies, although the potentially reactive electrophilic sites of these compounds were the sulfur atom and carbons at positions 3 and 5. The carbon atom at position 4 being adjacent to carbons C-3 and C-5, any chemical modification on these two electrophilic sites inducing a change in the electronic environment would be reflected in the ¹³C NMR chemical shift of C-4, thus making possible the monitoring of the reactivity with the peptides. Moreover, C-4 was a tertiary carbon bearing a hydrogen atom and thus we could make use of two-dimensional [¹H–¹³C]-NMR HSQC and HMBC data to elucidate the structures of the products formed, which would not have been possible in the case of the quaternary carbon atoms C-3 and C-5.

In the reactions carried out using classical reactivity conditions, 4-[¹³C]-MI or 4-[¹³C]-MCI were reacted with an excess of GSH (10 equiv.) or with an excess of Pep-Cys or Pep-Lys (2 equiv.) in a 6:4 (v/v) CD₃CN/H₂O mixture.

In the reactions carried out using the conditions of the peptide depletion test, an excess of 4-[¹³C]-MI or of 4-[¹³C]-MCI (2 or 10 equiv.) was reacted with the peptides, in a phosphate buffer solution in the case of GSH and Pep-Cys (0.1 M, pH 7.4 if GSH; 0.1 M, pH 7.5 if Pep-Cys), or in an ammonium acetate buffer solution in the case of Pep-Lys (0.1 M, pH 10.2). According to the peptide depletion protocol, the pH of the Pep-Lys reaction was set at 10.2 in order to increase the reactivity of the primary amine of the lateral chain of lysine (pK_a 10.43 ± 0.1). For the NMR experiments, the proportion of D₂O in the NMR tube needed to be, at least, a quarter of the volume of the reaction solution (i.e., 163 μL). In consequence, all buffer solutions used to dissolve the peptides were prepared in a way that the final pH of the reaction solution was the one mentioned above after addition of D₂O.

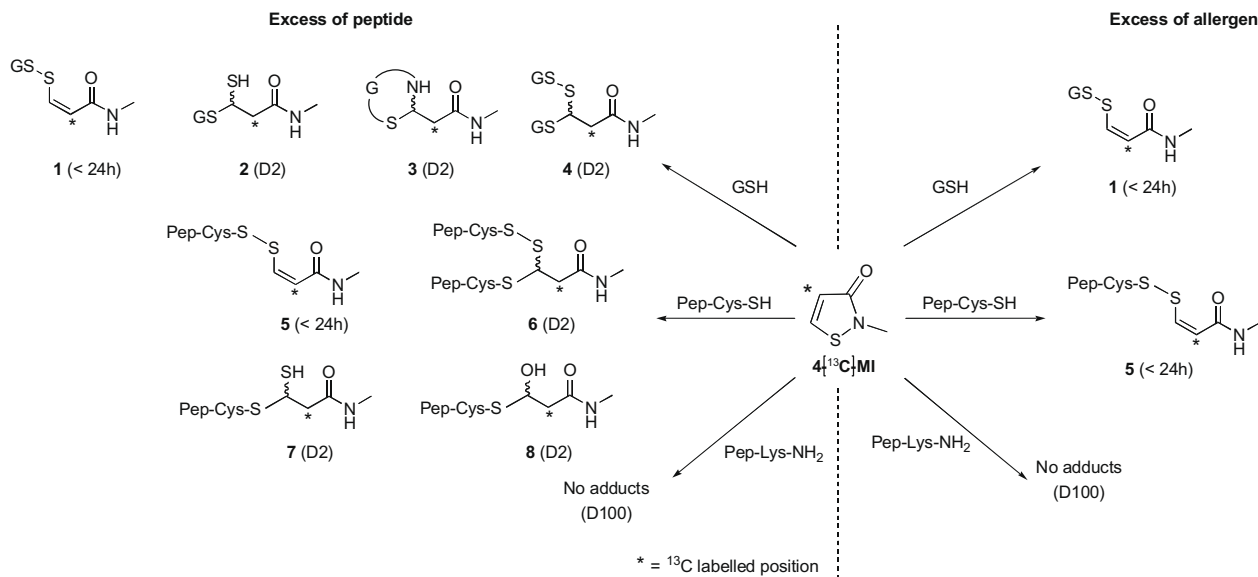
All the reactions were followed by ¹³C NMR and were stopped when there was complete disappearance of the 4-[¹³C]-N-methyl-isothiazolone, or after 100 days if that one was not completely consumed by the reaction or was in excess. The products formed were characterized by ¹H and ¹³C NMR data obtained by [¹H–¹³C]-HSQC and [¹H–¹³C]-HMBC experiments.

3.1. Reactivity of 4-[¹³C]-MI toward the peptides: classical reactivity and peptide depletion test conditions

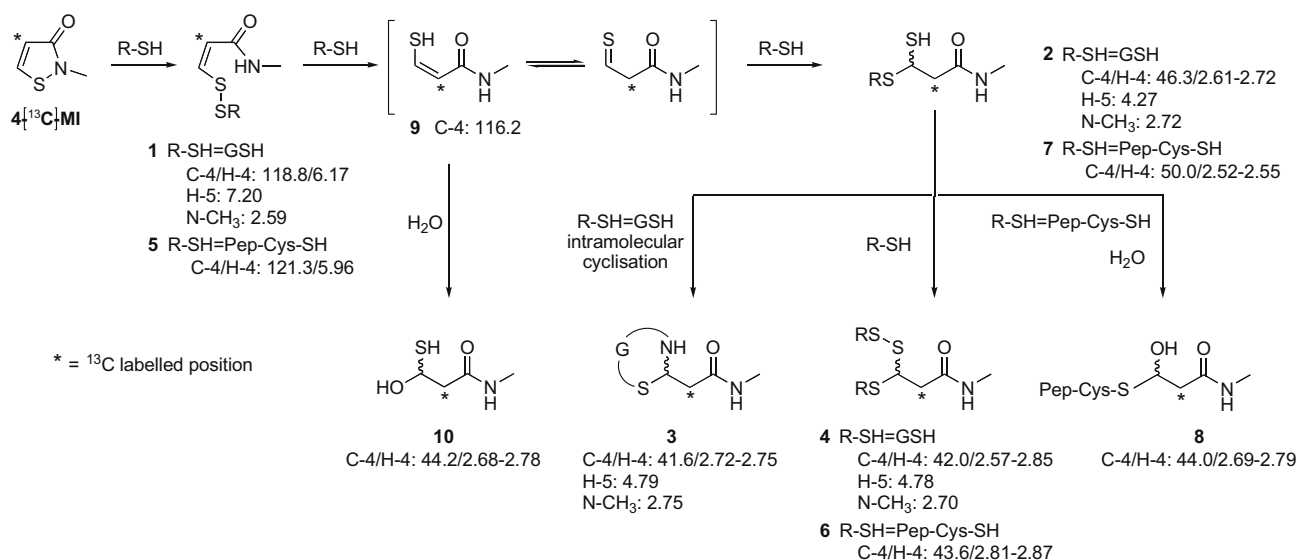
All the products identified when reacting 4-[¹³C]-MI with the peptides in the different experimental conditions are shown in [Scheme 1](#). Clearly, 4-[¹³C]-MI did not react with the lysine containing peptide Pep-Lys but reacted easily with the peptides containing the nucleophilic thiol group.

3.1.1. Reactivity of 4-[¹³C]-MI toward GSH and Pep-Cys

4-[¹³C]-MI showed the same kind of reactivity toward GSH and the cysteine peptide Pep-Cys in both experimental conditions tested. [Scheme 2](#) illustrates the reaction mechanisms that can account for the formation of products **1–10**. To begin with, a fast nucleophilic attack of the peptide thiol group on the electrophilic sulfur atom of the N-methyl-isothiazolone, followed by a ring opening, afforded adducts **1** and **5** in which the peptide was linked to the molecule through a disulfide bond. When the reaction was performed using an excess of hapten (peptide depletion test conditions), no further modification was observed after 24 h. [¹H–¹³C]-NMR experiments allowed then the characterisation of **1** and **5**



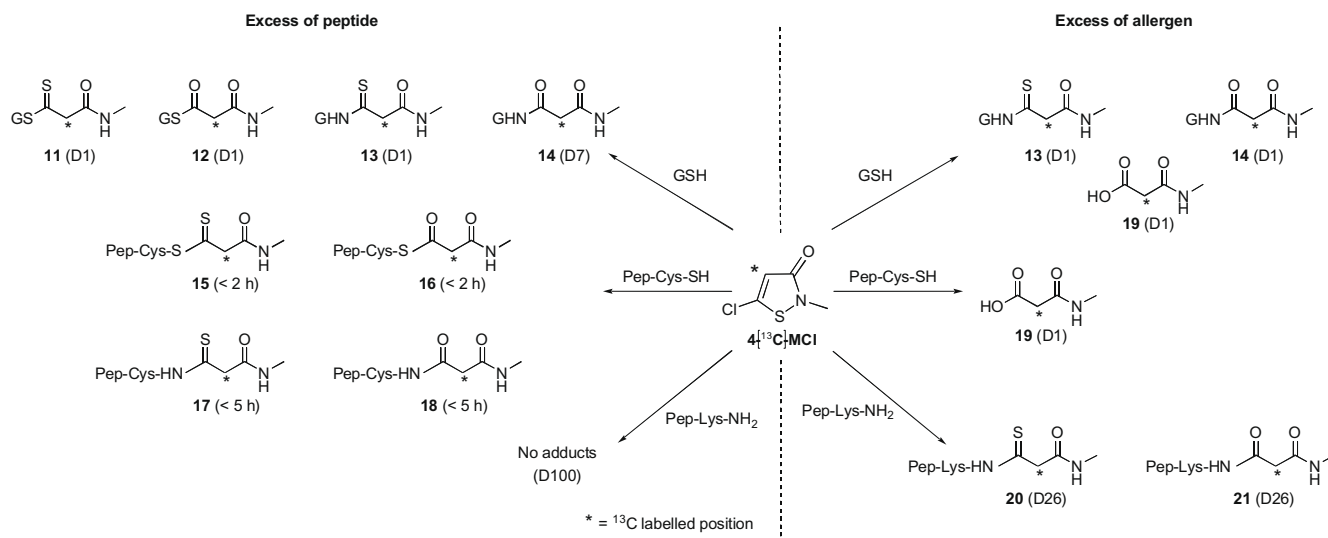
Scheme 1. Reactivity of 4-[¹³C]-MI toward the peptides in the different experimental conditions tested. Times indicated (D = day) do not refer to the kinetics of the reactions but to the instant in which the signals relevant to the products formed were detected in the NMR spectra.



Scheme 2. Reactivity of 4-[¹³C]-MI toward GSH and Pep-Cys: mechanistic interpretation for the formation of compounds 1–10. Characteristic ¹³C and ¹H NMR chemical shifts are indicated.

and showed the existence of long-range correlation signals between C-4/H-4 and H-5 and N-CH₃ (HMBC data). The C-4 δ values of 118.8 and 121.3 ppm, directly correlated by HSQC data to protons at 6.17 and 5.96 ppm, respectively, and the H-5 δ at 7.20 ppm confirmed that the C-4/C-5 double bond was preserved. However, in the presence of an excess of peptide (classical reactivity conditions), the reaction evolved further as signals corresponding to **1** (118.8 ppm) and **5** (121.3 ppm) disappeared after 2 days, together with a tiny signal at 116.2 ppm, and many new signals appeared in the 40–50 ppm region. Under these conditions, a new molecule of peptide could react with the disulfide bond and afford the thioacrylamide **9** in equilibrium with the thioxopropionamide form. The ¹³C NMR signal at 116.2 ppm corresponded indeed to intermediate **9** corroborating this hypothesis. The small signal at 116.2 ppm related to **9** was only observed in the reactivity studies with GSH and not in those using Pep-Cys, probably due to the

detection limits of the NMR method. Further reaction of **9**, highly electrophilic, with the peptide in excess resulted in the formation of adducts **2** and **7**. The C-4 δ values of 46.3 and 50.0 ppm, correlated by HSQC data to protons at 2.61–2.72 and 2.52–2.55 ppm, respectively, and the H-5 δ at 4.27 ppm were characteristic of a compound bearing a peptide unit and a thiol chemical group bound at C-5. Again, HMBC data confirmed the existence of long-range correlation signals between C-4/H-4 and H-5 and N-CH₃. In the case of adduct **2**, and due to the presence of a free nucleophilic amino group in GSH, the peptide linked at C-5 underwent an intramolecular cyclisation to give compound **3**, characterized with a higher δ value for H-5 and a lower δ value for C-4. Another possibility of evolution of **2** and **7** in the presence of an excess of peptide was the formation of a new disulfide bond through oxidation of the thiol chemical group at C-5 and further reaction with a new molecule of GSH or of Pep-Cys, giving products **4** and **6**,



Scheme 3. Reactivity of 4-[^{13}C]-MCI toward the peptides in the different experimental conditions tested. Times indicated (D = day) do not refer to the kinetics of the reactions but to the instant in which the signals relevant to the products formed were detected in the NMR spectra.

respectively, also with distinctive δ values. In addition, there could be reaction of the oxidised thiol group of **2** and **7** with the dimer of GSH (GSSG) or the one derived from Pep-Cys, both formed for instance in passing from **1** or **5** to thioacrylamide **9**. In the reaction using Pep-Cys we also observed, in the ^{13}C NMR spectra, adduct **8** resulting most probably from hydrolysis of **7**. Finally, product **10**, result of the hydrolysis of intermediate **9**, was just detected in the reactivity studies with GSH. As a final point, it is important to note that during all these reactions there was formation of diastereomers for many of the obtained adducts, this being reflected in the spectra by a splitting of the corresponding signals.

3.2. Reactivity of 4-[^{13}C]-MCI toward the peptides: classical reactivity and peptide depletion test conditions

Scheme 3 shows all the products identified when reacting 4-[^{13}C]-MCI with the peptides in the different experimental conditions tested. The nature of the adducts was different to that of those formed with 4-[^{13}C]-MI. However, it could be seen at a first sight that the kind of chemistry involved was somewhat similar in respect to the reactivity with thiol groups. On another hand, 4-[^{13}C]-MCI was able to react with the amino group of the lateral chain of lysine but also with other amino groups present on the peptides.

3.2.1. Reactivity of 4-[^{13}C]-MCI toward GSH and Pep-Cys

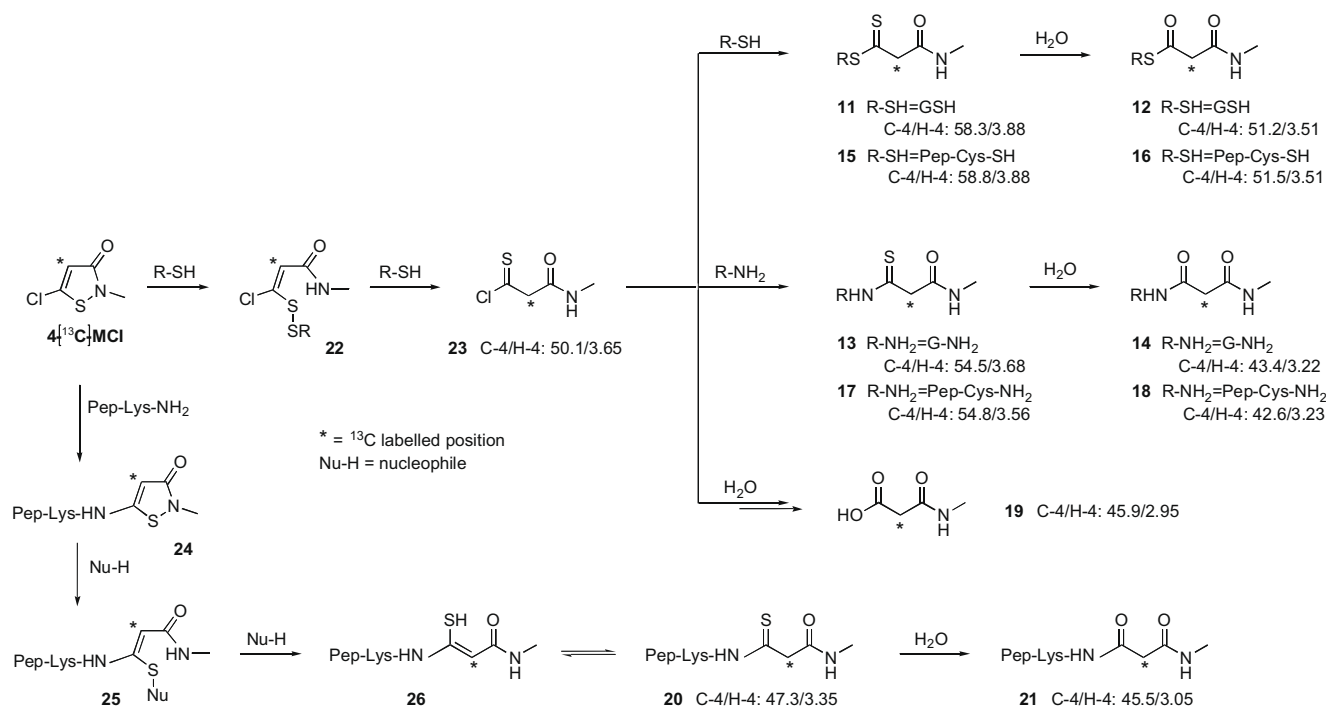
The reactivity of 4-[^{13}C]-MCI in the presence of an excess of GSH or of Pep-Cys (classical reactivity conditions) was very fast, its characteristic ^{13}C signal at 114.0 ppm disappearing in a few hours. New ^{13}C peaks at 43.4, 51.2, 54.5 and 58.3 ppm appeared in the reaction with GSH, and at 42.6, 51.5, 54.8 and 58.8 ppm in the reaction with Pep-Cys. The reaction mechanism(s) that could account for the formation of the different products is outlined in Scheme 4. Firstly, a mechanism comparable to the one producing the ring opening of 4-[^{13}C]-MI by reaction with thiol groups could now afford the thioacyl chloride derivative **23**, highly electrophilic. This intermediate was clearly observed in the reaction carried out with Pep-Cys in which a signal at 50.1 ppm appeared after only 1 h of reaction. The HSQC data associated this carbon δ value to a characteristic proton at 3.65 ppm. The thioacyl chloride chemical function was then able to react with any nucleophile present in the reaction mixture. Reaction with thiol groups of the peptides afforded dithioester derivatives such as **11** (58.3/3.88 ppm) and **15**

(58.8/3.88 ppm), that could be partly hydrolysed and form **12** (51.2/3.51 ppm) and **16** (51.5/3.51 ppm), respectively. But also, reaction with the nucleophilic amino groups present in both peptides was possible and adducts of the thioamide kind such as **13** (54.5/3.68 ppm) and **17** (54.8/3.56 ppm) were observed. The δ value of 54.8 ppm, for example in the case of **17**, was characteristic of a thioamide adduct obtained by reaction of **23** with the free amino group of the lateral side chain of the arginine residue of Pep-Cys. As for the dithioester derivatives **11** and **15**, the thioamides **13** and **17** could be partly hydrolysed and adducts **14** (43.4/3.22 ppm) and **18** (42.6/3.23 ppm) were noticed. An example of monitoring is shown in Fig. 2 where it can be easily seen the decrease in the intensity of the ^{13}C peak of the thioacyl chloride **23**, in favour of the peaks corresponding to the adducts. In view of these results, the behaviour of GSH and Pep-Cys toward 4-[^{13}C]-MCI was identical and the same kind of adducts were formed.

In the presence of an excess of hapten (peptide depletion test conditions), not enough peptide was available to follow all the reactivity described above, and only the strongest nucleophilic chemical function was able to react with **23**. Thus, only the amino group of GSH, much more nucleophilic toward thioacyl chloride derivatives than the thiol chemical function of both peptides or the amino group of the Pep-Cys arginine residue, reacted with **23** to form adduct **13**, partly hydrolysed to **14**. The hydrolysis product of **23** was also observed. Chemical shifts of **19** at 45.9 and at 2.95 ppm were indeed characteristic of a methylene group bearing a carboxylic acid and an amide chemical function.

3.2.2. Reactivity of 4-[^{13}C]-MCI toward Pep-Lys

The experiments studying the reactivity of 4-[^{13}C]-MCI in the presence of an excess of Pep-Lys did not show the formation of any product nor adduct. A possible explanation could be that the amino group could not behave mechanistically as the thiol group and therefore the reaction mechanisms described to here that resulted from a first reaction between the thiol chemical function and the sulfur atom of the *N*-methyl-isothiazolone, leading to the opening of the heterocycle, could not be applied in the case of Pep-Lys. However, a reactivity was observed when using an excess of 4-[^{13}C]-MCI. Using the peptide depletion test experimental conditions, the pH of the solution was set at 10.2 to increase the nucleophilic character of the free amino group of the lateral chain of lysine, and thus favouring its reactivity. This was not the case when the peptide was in excess and thus, in reality, the experimental



Scheme 4. Reactivity of 4-[¹³C]-MCI toward GSH, Pep-Cys and Pep-Lys: mechanistic interpretation for the formation of compounds **11–21**. Characteristic ¹³C and ¹H NMR chemical shifts are indicated.

conditions were not favourable for a nucleophilic attack of the free amino group on 4-[¹³C]-MCI.

The thioamide adduct **20** (47.3/3.35 ppm) together with its hydrolysis derivative **21** (45.5/3.05 ppm) were identified. Most probably, the reaction between 4-[¹³C]-MCI and Pep-Lys started by a nucleophilic addition of the free amino group of the lateral chain of lysine on the C-5 electrophilic carbon atom of 4-[¹³C]-MCI (Scheme 4), followed by elimination of chlorine. The isothiazolone **24** with the peptide linked at C-5 and resulting from this reaction could further follow another nucleophilic attack by another nucleophile present in the reaction mixture leading to the opening of the cycle and to the subsequent formation of adduct **20**. Such a reaction resulting from an attack at the sulfur atom of nucleophilic groups other than thiols has already been reported (Crow and Leonard, 1965) even if in our case the precise nature of this nucleophile is not known. The proposed reaction mechanism was only hypothetical. During these reactivity experiments we did not have any experimental proof nor identified any intermediate allowing the validation of the mechanism proposed. Nevertheless, we did show in earlier studies using MCI ¹³C labelled at positions C-3, C-4 and C-5, that the transition through intermediates like **24** and **26** was probable (Alvarez-Sánchez et al., 2003). Reaction studies with imidazole, used as a simple model for histidine, proved the addition–elimination reaction at position 5 of MCI. The presence of imidazole at position 5 forming a **24**-like adduct was corroborated by HMBC experiments. Moreover, reaction studies with butyl amine, used as a simple model of lysine, confirmed the addition–elimination reaction already observed with imidazole and the formation of a **24**-like adduct, but also the opening of the isothiazolone ring with the formation of a **26**-like adduct, in equilibrium with the thioamide form. In addition, reactivity studies of labelled MCI in a semi organic medium (CH₃CN/PBS) with a model peptide that included all natural amino acids excepted for cysteine confirmed, by the use of ¹H and ¹³C NMR data of the adducts observed, the formation of a **24**-like adduct with histidine and the formation of amino adducts of the **20** thioamide type and of the **21** amide type with lysine (Alva-

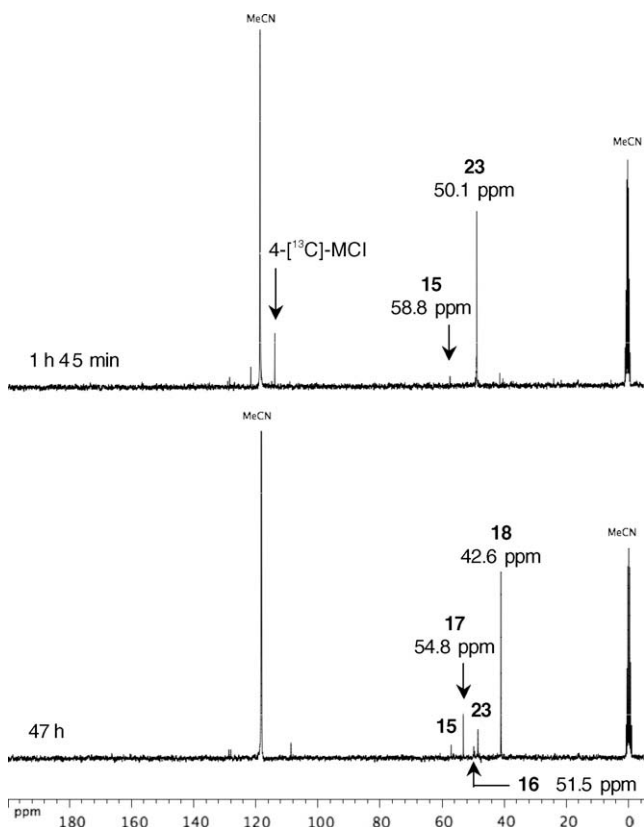


Fig. 2. Reaction between 4-[¹³C]-MCI and Pep-Cys followed by one-dimensional ¹³C NMR. Registered spectra at 1 h 45 min and at 47 h of reaction showed the involvement of the thioacyl chloride intermediate **23** in the formation of the adducts.

rez-Sánchez et al., 2004a). Thus, even if the mechanism shown in Scheme 4 was hypothetical, it was possible that adducts **20** and **21** were formed in this way, our preceding studies giving support to the probable formation of intermediates **24–26**.

4. Discussion

A major goal in the skin sensitization field is to develop in vitro/ in silico tools for the screening of chemicals for their skin sensitization hazard and potency, and to devise alternative approaches to replace in vivo assays. A better understanding of the skin sensitization process at the molecular level is particularly helpful to support this goal. The in vitro peptide binding based assay we developed for the screening of chemicals and skin sensitization hazard (Gerberick et al., 2004, 2007), in spite of using experimental conditions different than those expected in vivo, needed to be based on a similar hapten–peptide binding chemistry in order to be somehow relevant. In this work reactivity studies of MI and MCI, classified by the LLNA as weak and strong allergens, respectively, toward the three peptides used in the peptide binding assay were carried out with the purpose of validating the hapten–peptide reactivity concept under the specific experimental conditions used in the assay.

After synthesis and ^{13}C labelling of MI and MCI, reaction mechanisms and adduct formation toward GSH, Pep-Cys and Pep-Lys were investigated by one- and two-dimensional NMR, under the screening assay conditions (excess of hapten), and the ones expected to happen in vivo (excess of peptide). It is essential to note here that the reaction times given through all the manuscript did not correspond to kinetic studies of the formation of the reaction products but to the time necessary to detect them distinctly by NMR. Thus, it could not be excluded that the formation of the NMR identified reaction products happened before the time necessary to detect them. The chemistry observed was similar to the one reported with model nucleophiles (Alvarez-Sánchez et al., 2003). However, it was a complex chemistry as the multi-step reactions observed, leading to the formation of various adducts with different structures, were not the result of a simple reaction between one molecule of hapten and one molecule of peptide. To that respect, following the peptide depletion rather than the formation of many diverse adducts is more adapted to a screening in vitro test.

4- ^{13}C -MI was found to be reactive toward the thiol containing peptides GSH and Pep-Cys, but completely inert toward Pep-Lys. Under both conditions tested, an initial nucleophilic attack of the peptide thiol group at the electrophilic sulfur atom of the *N*-methyl-isothiazolone was followed by a ring opening to afford an acrylamide adduct in which the peptide was linked to the molecule through a disulfide bond (Scheme 2). In the presence of an excess of peptide (expected in vivo situation), this intermediate could evolve further and react to form many different adducts while in the presence of an excess of hapten (peptide reactivity test) the reaction stops after this first step. The formation of potential hapten–peptide complexes is thus driven by this initial reaction. For the purpose of assessing the chemical reactivity of MI toward nucleophilic peptides, conditions used in the peptide reactivity assay allow to consider the first key step initiating the formation of adducts. These results were in agreement with the depletions measured for the peptides treated with MI. A significant depletion was observed for GSH (73%) and Pep-Cys (98%) (unpublished data) whereas an irrelevant Pep-Lys depletion was evidenced (3%), corresponding well with the lack of reactivity of MI toward this peptide. The higher Pep-Cys depletion observed could probably be related to the longer incubation time when using this peptide, 24 h instead of 15 min for GSH.

4- ^{13}C -MCI was shown to be very reactive toward the thiol containing peptides GSH and Pep-Cys. The reaction mechanism(s) started as described for 4- ^{13}C -MI with the difference that a new intermediate **23**, of the thioacyl chloride type, was formed after ring opening of the cycle and further attack of a molecule of thiol peptide (Scheme 4). This newly formed highly electrophilic thioacyl chloride intermediate can then react with the different nucleophiles present in the reaction medium. Thus when the peptide was in excess, two types of adducts were formed depending on the nucleophilic group(s) present on the peptide. In the presence of an excess of GSH and Pep-Cys, a competition was observed between the thiol and the amino groups leading to the formation of four different adducts, **11–14** and **15–18**, respectively. However, in the presence of an excess of MCI only adducts **13** and **14**, resulting from the reaction between the free amino group of GSH and **23**, were observed. This result showed that, in the experimental conditions used in the screening assay, the free amino group of GSH had a stronger nucleophilic character toward thioacyl chloride derivatives than the GSH thiol group. However, these two nucleophiles are also in competition with water to afford the carboxylic acid **19** deriving from hydrolysis of the thioacyl chloride derivative. In the case of Pep-Cys the situation is quite different as the guanidine residue present in the peptide has a low nucleophilic potential due to its pK_a value and only the hydrolysis product **19** was observed. We could therefore conclude that the formation of a thioacyl chloride intermediate played a central role in the reactivity of 4- ^{13}C -MCI toward GSH and Pep-Cys, whatever the conditions used even if leading to very different products depending of the reaction conditions.

In the screening test conditions, using an excess of allergen to ensure an optimal peptide depletion, some reactivity was also observed between 4- ^{13}C -MCI and Pep-Lys. In the assay the pH of the incubating medium was set at the pK_a of the reactive amino acid side chain, that means pH 10.2 for the Pep-Lys reactions. Under this pH the reaction potential was thus maximized as the free amino group of the side chain of the lysine residue was not protonated and its nucleophilic character was enhanced. 4- ^{13}C -MCI reacted through a different mechanism (Scheme 4) to form a thioamide kind adduct **20** and its hydrolysis derivative **21**.

For the purpose of assessing the chemical reactivity of MCI toward nucleophilic peptides, conditions used in the peptide reactivity assay allow here again to consider the first key step leading to the formation of adducts. It is striking to note that in the case of Pep-Cys, the only compound detected was the hydrolysis product **19** which is no more an adduct but signs the formation of the thioacyl chloride resulting of the reaction of MCI with the thiol residue. This confirms that detection of adducts for the quantification of hapten–peptide reactivity could be, in some cases, misleading and that a measure of the peptide depletion could be more accurate. These results were also in good agreement with the depletions measured for the peptides treated with MCI (unpublished results).

In view of the results reported, the *N*-methyl-isothiazolones showed in general the same kind of reactivity toward the peptides tested in the screening assay conditions (excess of hapten) and in the ones expected to happen in vivo (excess of peptide). Thus, the reactivity observed when the peptide binding assay conditions were employed could be considered representative of the one observed in in vivo approaching conditions. The only exception to this statement was the reactivity observed between an excess of 4- ^{13}C -MCI and Pep-Lys due to the pH of the incubating medium, set to 10.2 in order to take advantage of the nucleophilicity of the amino group of the lateral chain of lysine, being not representative of the pH in vivo.

Peptide depletion studies carried out with MI and MCI separately (unpublished data) indicated a strong reactivity of both

compounds toward the peptides containing the nucleophilic thiol group but no significant differences in the behaviour of both of them were evidenced. It seemed therefore not possible to correlate the strong allergenic potential of MCI and the weak sensitizing potential of MI with the quantification of the depletion of thiol containing peptides. However, the Pep-Lys depletion results distinguished the behaviour of MI and MCI, no depletion being observed for the weak sensitizer MI and 35% depletion observed for the strong allergen MCI. Being in agreement with this, in our reactivity studies we did observe only for MCI the formation of adducts with Pep-Lys and this when the conditions of the depletion peptide test were used. Hence, the use of the reactivity data with Pep-Lys, even if at a pH that is far from the physiological pH, could maybe make the difference in distinguishing the sensitizing potential of both isothiazolones. It should be mentioned that such lysine adducts have been evidenced after reaction with human serum albumin (Alvarez-Sánchez et al., 2004b) and could therefore be considered as relevant.

To conclude, the formation of the several adducts detected by ^{13}C NMR matched relatively well with the depletions of the peptides measured. Initially it seemed therefore reasonable to study the hapten–peptide reactivities by analysing the percentage of chemical modifications on the peptides. Nevertheless, and importantly, the studies reported in here also showed that the hapten–peptide reactivity was not restricted to the unique formation of adducts. Some of these adducts were reactive and could form hydrolysis products no more covalently bound to the peptide. This suggested, on haptens to which consumers are exposed and which are clinically relevant, that a quantification of adducts could be difficult and therefore misleading, while a measure of the peptide depletion is a simpler approach to the assessment of the reactivity of these molecules toward nucleophiles. Therefore, this study strengthens the peptide reactivity concept as well as the choice made to look at peptide depletion rather than at adduct formation strictly.

Conflict of interest statement

None declared.

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